

# MICROBIOLOGICAL STUDY OF ACID MINE WATERS: PRELIMINARY REPORT

J. MEHSEN JOSEPH

*Department of Bacteriology, University of Toledo, Toledo, Ohio*

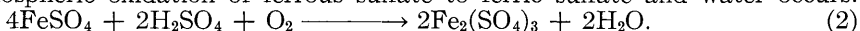
Waters issuing from bituminous mines may be either acid or alkaline. The acid mine waters are objectionable; their disposal or abatement is highly desirable. Many agencies are concerned in the reduction of this important industrial waste for several reasons: pollution with acid wastes renders streams extremely acidic, pH 2.0 to pH 4.0, which results in the annihilation of the majority of commonly encountered micro- and macroorganisms in water; high concentrations of ferric, ferrous, and sulfate compounds are hazardous to children using such streams as bathing areas; the aesthetic quality of these streams is in a deplorable condition due to the deposition of ferric hydroxide in the channels of the streams.

## FORMATION OF ACID MINE WATER

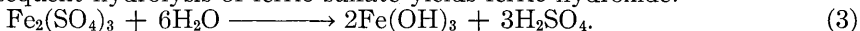
The oxidation of sulfur and sulfur-bearing compounds in coal seams results in the formation of large quantities of acid compounds available for solution in water. It is assumed that ferric disulfide is oxidized to soluble ferrous sulfate and sulfuric acid:



When these acid wastes enter streams they are exposed to atmospheric conditions and atmospheric oxidation of ferrous sulfate to ferric sulfate and water occurs:



The subsequent hydrolysis of ferric sulfate yields ferric hydroxide:



Ferric hydroxide in conjunction with hydrated iron oxides is precipitated in the stream's course to impart a rusty color.

Carpenter and Herndon (1933) and Hodges (1938) have reported analyses of this type of drainage which vary widely from one stream to another. The analysis of a stream gave the following results; pH 2.0; total acidity, 47,800 ppm; sulfates, 41,700 ppm; total iron, 12,270 ppm. The author has reported the following variations for streams having a pH range from 2.0 to 2.9: total acidity, 196–106,000 ppm; total residues, 7,700–40,000 ppm; calcium, 145–300 ppm; magnesium, 20–55 ppm; sulfate, 100–18,300 ppm; ferric iron, 30–3,900 ppm; and ferrous iron, 20–1,200 ppm.

Numerous attempts have been made to abate or modify acid drainage issuing into many streams. Sealing of abandoned mines has been instituted to reduce the oxygen concentration with the hope that less oxidation of sulfur compounds will ensue, resulting in a smaller amount of available acid compounds for solution in water. Chemical treatment to modify or rid water of objectionable compounds has been studied. Since such treatment is expensive and results in the accumulation of large amounts of sludge, it is not feasible.

Colmer and Henkle (1947) have approached the problem from a micro-biological point of view. They maintain that microorganisms are in part responsible for the formation of large quantities of sulfuric acid and ferric hydroxide. Therefore, studies were undertaken to determine the role of microorganisms in the formation of acid mine waters. They were able to isolate two significant organisms; one was an unidentified bacterium involved in the oxidation of ferrous sulfate to ferric sulfate, and the other was *Thiobacillus thiooxidans* which is capable of oxidizing sulfur compounds to sulfuric acid. It is suggested that the study of the relationship

of microorganisms to the formation of sulfuric acid might provide a more effective method for the abatement of objectionable acid wastes.

Since conditions of extreme acidity and hyperconcentration of iron compounds provide a profound adverse environment for the survival of a great majority of micro- and macroorganisms indigenous to streams, the author has instituted the investigation of the diversity of genera and species tolerating such conditions.

In an attempt to review the literature pertaining to the microbiological assay of acid mine waters it becomes apparent that such information is entirely lacking. A few theses and articles of many kinds were examined, but data pertinent to the author's work are not accessible.

These studies were first begun to determine the viability of *Escherichia coli*, the organism used as the index of fecal pollution, in acid mine streams, but were later merged into a study of all viable microbes. The task at hand was to isolate, cultivate, observe, and attempt identification of the various kinds of organisms capable of tolerating conditions existing in acid waters and to observe the direct or indirect effects of acid waters upon the existing microorganisms. A preliminary study was also made for the special groups, i.e., colon-typhoid, sulfur bacteria, iron bacteria, actinomycetes, and fungi.

#### MATERIALS AND METHODS

*Microflora.* Samples were collected from acid streams in northern West Virginia and Pennsylvania in previously sterilized (autoclaved) sampling bottles by submerging the mouths of the bottles against the current of the stream (most aseptical method), and filled to capacity to eliminate any air space. The presence of sufficient oxygen would result in the oxidation of ferric sulfate to ferric hydroxide and sulfuric acid. Analyses of all samples were made as soon as possible after collection.

The above water samples were plated on various agars suspected of supporting the growth of a few or a single organism. Various types of broth combinations were inoculated with aliquots of mine water. Streaks were made for these broth tubes onto agar in an attempt to procure pure cultures of the growing organisms. Pure cultures were numbered and transferred to fresh media at intervals in order to maintain identity and viability. The preparation of suitable media proved to be the most difficult task.

In order to isolate special group organisms, elective cultural methods were employed. All waters were inoculated into the synthetic medium of Waksman and Starkey (1922) for the cultivation of sulfur bacteria, artificial mine water medium for the cultivation of iron bacteria, peptone malt agar for fungi, and potato glycerol agar for actinomycetes.

*Diatom study.* In collecting samples of diatoms, pyrex resistant glass was used in order to reduce the deteriorating effects induced by the absorption of alkali from ordinary glass. On each trip to the mine streams definitely marked spots were examined continually so as to thoroughly study the algae present. Subsequently, various other areas were picked and thoroughly studied. After a great number of samples were collected from numerous areas, no cross-sectional data of the diatom flora were obtained. Precise notes were taken as to the time of sampling, the place and the exact spot so that in case further study is necessitated one may return to the exact location for sampling. All of the above data were placed on each sampling bottle, and if worthy of preservation, it was recorded in a record book. Samples were taken by the removal of surface mud and scraping from rocks and twigs on the stream bed.

Of great importance is the immediate examination of samples, since algae tend to deteriorate rapidly. If samples were to be stored for any length of time, special methods of preservation were employed, namely, fixation with King's fluid, picrosulfuric acid, or osmic acid. One of the best methods for the study of living

diatoms is by the hanging drop mount preparation. Motility and similar functions performed by delicate structures are very difficult to preserve, thus, immediate observation is recommended and almost indispensable.

The internal structure of diatoms was not considered in their identification. However, the ornamental sculpturing of their cell wall, their shape, and their size were utilized to accurately determine genus and species names.

The method employed for elucidating cell wall sculpturing was the siliceous corrosion method. This procedure may be outlined briefly. Treat the material containing diatoms with concentrated HCl until effervescence ceases. Allow material to remain in HCl for two days to remove any debris adhering to the diatoms, and then filter through coarse muslin. Centrifuge the filtrate and wash the remaining precipitate several times with water. The washed precipitate is treated with concentrated  $H_2SO_4$  and a small crystal of  $K_2Cr_2O_7$  to char or dissolve any organic material present. Repeat the  $H_2SO_4$ -bichromate treatment several times. When the diatoms are scrupulously cleaned, they are infiltrated with canada balsam and mounted on microscope slides. The slides thus prepared are used for taxonomic study.

*Microfauna.* Isolation and identification of the organisms comprising the microfauna were based entirely upon the microscopic study of cellular morphology. Various water samples, atrocious surface deposits, and surface soils were collected and examined. Cultivation of this group was not attempted, since microscopic observation proved sufficient for identification. Seasonal examinations were made in order to account for any possible variation of the microfauna with the season.

## RESULTS AND DISCUSSION

### *Microflora*

*Bacteria.* By the exploitation of every method for isolation, either original or duplicated, 40 species of bacteria were obtained in pure cultures. Of the 40 species, 14 were gram positive, spore-forming rods belonging to the genus *Bacillus*, 2 were gram-negative rods belonging to the genera *Escherichia* and *Aerobacter*, and 20 were gram-positive cocci belonging to the genera *Micrococcus* and *Sarcina*. The remaining species were members of the genera *Thiobacillus*, *Crenothrix*, and *Microsporium*.

Colmer and Henkle (1947) have reported frequent isolations of *Thiobacillus thiooxidans* from acid mine streams which provide confirmative evidence. Ellison (1919) reported the isolation of *Microsporium desulphuricans* and *Vibrio desulphuricans* from surface mud in acid mine water.

Table 1 illustrates very clearly the presence of bacteria in a paucity of numbers in acid mine streams, and table 3 shows that the members are equally divided between the genera *Bacillus* and *Micrococcus*.

*Fungi.* It is interesting to note that fungi are not as well represented as one would anticipate in view of the existing acid conditions conducive to their growth. Organisms of the genera *Aspergillus*, *Helminthosporium*, *Penicillium*, *Trichoderma*, *Cladosporium*, *Alternaria*, and *Trichothecium* were isolated and identified. The relative quantitative plate counts for fungi and bacteria may be seen in table 1.

*Actinomyces.* Members of the genus *Actinomyces* were not isolated directly from acid mine streams, but the examination of soils moistened with mine water seepage showed their presence.

*Diatoms.* Thorough investigation of the abominable brownish and greenish-brown deposits on the stream beds revealed the presence of myriads of diatoms having an almost ubiquitous distribution. *Navicula viridis* appeared to be the only existing form, but by very meticulous and effectual methods many other genera and species were identified. The multitude of diatoms in every acid mine stream examined has led the author to suggest that these organisms may be

used as an index of acidity. A total of 8 genera and 18 species of the class Bacillarieae have been characterized, and are recorded in table 2. It is beyond doubt that diatoms are the most abundant forms of life to be found in acid mine streams.

TABLE 1  
*Quantitative plate counts for acid mine streams and soils along their banks.*

| Stream | pH   | Bacterial Count<br>per ml | Fungal Count<br>per ml | Actinomyces Count<br>per gm of soil |
|--------|------|---------------------------|------------------------|-------------------------------------|
| A      | 2.0  | 4,800                     | 90,000                 | 3,600                               |
| B      | 2.4  | 18,000                    | 60,000                 | 6,400                               |
| C      | 2.5  | 39,000                    | 120,000                | 13,000                              |
| D      | 2.8  | 24,000                    | 118,000                | 7,000                               |
| E      | 3.0  | 180,000                   | 160,000                | 13,000                              |
| F      | 3.15 | 150,000                   | 190,000                | 21,000                              |
| G      | 3.3  | 83,000                    | 180,000                | 7,500                               |
| H      | 3.9  | 470,000                   | 110,000                | 16,000                              |
| I      | 4.0  | 560,000                   | 590,000                | 26,000                              |
| J      | 4.0  | 340,000                   | 640,000                | 18,000                              |

TABLE 2  
*Diatoms isolated from acid mine streams which have been identified.*

|                        |                               |                          |
|------------------------|-------------------------------|--------------------------|
| 1. Navicula viridis    | 7. Surirella biseriata        | 13. Cocconeis placentula |
| 2. Navicula lanceolata | 8. Surirella saxonica         | 14. Cocconeis pediculus  |
| 3. Navicula oblonga    | 9. Denticula spp.             | 15. Cocconeis spp.       |
| 4. Navicula spp.       | 10. Nitzschia sigmoidea       | 16. Synedra ulna         |
| 5. Navicula spp.       | 11. Homoeocladia vermicularis | 17. Meridion circulare   |
| 6. Navicula spp.       | 12. Homoeocladia spp.         | 18. Rhoisphenia curvata  |

TABLE 3  
*Microorganisms isolated from acid mine waters.*

|                 |                                       |                                       |
|-----------------|---------------------------------------|---------------------------------------|
| BACTERIA:       | <i>Bacillus subtilis</i>              | <i>M. pyogenes</i> var. <i>aureus</i> |
|                 | <i>Bacillus</i> spp.                  | <i>M. varians</i>                     |
|                 | <i>B. lentus</i>                      | <i>M. desulphuricans</i>              |
|                 | <i>B. cereus</i>                      | <i>Escherichia coli</i>               |
|                 | <i>B. mycoides</i>                    | <i>Aerobacter aerogenes</i>           |
|                 | <i>Micrococcus</i> spp.               | <i>Thiobacillus thioparus</i>         |
|                 | <i>M. pyrogenes</i> var. <i>albus</i> | <i>T. thiooxidans</i>                 |
|                 |                                       | <i>Crenothrix polyspora</i>           |
| FUNGI:          | <i>Aspergillus</i> sp.                | <i>Alternaria</i> sp.                 |
|                 | <i>Trichoderma</i> sp.                | <i>Penicillium</i> sp.                |
|                 | <i>Helminthosporium</i> sp.           | <i>Trichothecium</i> sp.              |
|                 |                                       | <i>Cladosporium</i> sp.               |
| ACTINOMYCES:    | Several unidentified species          |                                       |
| DIATOMS:        | (see table 2)                         |                                       |
| PROTOZOA:       | <i>Amoeba proteus</i>                 | <i>E. mutabilis</i>                   |
|                 | <i>Euglena viridis</i>                | <i>Paramecium caudatum</i>            |
| TROCHELMINTHES: | Rotifers                              |                                       |

### *Microfauna*

Animal life fares no better in these acid streams, since only the phyla Protozoa and Trochelminthes were identified.

Protozoa were represented by *Euglena viridis* and *Euglena mutabilis* of the

class Mastigophora, by *Paramecium* of the class Ciliata, and by *Amoeba* of the class Sarcodina. *Euglena viridis* is *per se* as abundant as the entire group of diatoms, for they are very acid-tolerant organisms. Trochelminthes were found very sparingly and only as rotifers. Lackey (1938) has reported the existence of rotifers, ciliates, and flagellates in acid waters which provides a corollary to these observations.

In passing it might be mentioned that higher animal and plant forms than those reported here are not to be found in acid mine waters, save a few plant forms. Thus, one can readily see that these waters constitute a very objectionable class. Even though conditions in acid mine streams appear highly adverse for the viability of microorganisms, Joseph (1951) has shown by means of longevity studies and actual isolation of *Escherichia coli* that the transmission of disease is still possible.

#### SUMMARY

The most highly acid streams will support the growth of a few microscopic organisms.

Bacteria are represented by the genera *Bacillus*, *Micrococcus*, *Sarcina*, *Escherichia*, *Aerobacter*, *Thiobacillus*, *Crenothrix*, and *Microsporium*. The bacteria are almost equally divided among the genera *Bacillus* and *Micrococcus*, but their abundance relative to their numbers in alkaline waters indicates an intensive detrimental effect of acid mine water. This is also borne out by the fact that variant or aberrant forms of the surviving organisms are frequently encountered. *Escherichia coli* is capable of surviving acid mine waters, therefore, its tolerance is indicative of the ability of the typhoid bacillus to also be tolerant and probably present.

Fungi are relatively abundant and are represented by seven genera. Quantitative plate counts for fungi range from 60,000 to 640,000 per ml, but these count are much lower than one would anticipate. It is also interesting to note that Actinomyces were not isolated from acid mine waters, but were present in the soil along the banks of the streams. Quantitative plate counts for Actinomyces range from 3,600 to 26,000 per gram of soil.

Diatoms are very abundant in acid mine streams, and are represented primarily by *Navicula viridis*. The author suggests the use of this organism as an index of acid conditions, since little skill is required for its recognition. A total of 8 genera and 18 species of diatoms have been isolated from various acid streams and identified.

There is a great reduction in the microfauna of acid mine waters, since they are shy of every phylum, except for a few members of the phyla Protozoa and Trochelminthes. Protozoa are represented by *Euglena viridis*, *Euglena mutabilis*, *Amoeba*, and *Paramecia*. *Euglena viridis* is the most abundant organism, and its association with acid waters indicates its use as an index for acidity. Trochelminthes are represented only by one member, rotifers.

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